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Tyrosine Hydroxylase is expressed during early heart development and is required for cardiac chamber formation

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Running Title: Tyrosine Hydroxylase in Heart Development

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Abstract

Aims: Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in catecholamine biosynthesis. Whereas the neuroendocrine roles of catecholamines postnatally are well known, the presence and function of TH in organogenesis is unclear. The aim of this study is to define the expression of TH during cardiac development and to unravel the role it may play in heart formation.

Methods and Results: We studied TH expression in chick embryos by whole mount *in situ* hybridization and by quantitative reverse transcription-polymerase chain reaction (RT-qPCR), and analyzed TH activity by high-performance liquid chromatography (HPLC). We used gain- and loss-of-function models to characterize the role of TH in early cardiogenesis. We found that TH expression was enriched in the cardiac field of gastrulating chick embryos. By stage 8, TH mRNA was restricted to the splanchnic mesoderm of both endocardial tubes and was subsequently expressed predominantly in the myocardial layer of the atrial segment. Overexpression of TH led to increased atrial myosin heavy chain (AMHC1) and T-box 5 gene (Tbx5) expression in the ventricular region, and induced bradycardia. Similarly, addition of L-3,4-dihydroxyphenylalanine (L-DOPA) or dopamine induced ectopic expression of cardiac transcription factors (cNkx2.5, Tbx5) and AMHC1, as well as sarcomere formation. Conversely, blockage of dopamine biosynthesis and loss of TH activity decreased AMHC1 and Tbx5 expression, whereas exposure to retinoic acid induced TH expression, in parallel to that of AMHC1 and Tbx5. Concordantly, inhibition of endogenous retinoic acid synthesis decreased TH expression as well as that of AMHC1 and Tbx5.

Conclusions: TH is expressed in a dynamic pattern during the primitive heart tube formation. TH induces cardiac differentiation *in vivo* and it is a key regulator of the heart patterning, conferring atriogenic identity.

Introduction

Catecholamines are hormones/neurotransmitters known to influence cardiovascular and endocrine physiology postnatally and processes such as movement, learning and emotional behaviour. TH is the first and rate-limiting enzyme in catecholamine biosynthesis. TH catalyzes the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which generates dopamine by the action of aromatic amino acid decarboxylase (AAADC). Subsequently, dopamine can be converted to noradrenaline, by the dopamine beta hydroxylase (DBH), and adrenaline in the mature nervous system and adrenal glands (Fig. 1A)¹. Despite the well-characterized biological functions of catecholamines in postnatal organisms, very little is known about their function in embryonic development prior to neuronal differentiation. Early reports of the presence of the pathway members, and their pharmacological and genetic interference, revealed roles in gastrulation and early organogenesis². Indeed, null-mutations of TH and DBH caused embryonic lethality³⁻⁵ due to apparent heart failure. Although studies of the TH-null mice revealed a functional role of TH in maintaining oxygen homeostasis at mid-gestation stages^{6,7}, the role of TH in early cardiogenesis remains unexplored.

In vertebrates, the heart is initially formed by the fusion of the two bilateral endocardial tubes arising in the lateral plate splanchnic mesoderm. The resulting primitive heart tube, located at the ventral midline of the organism, undergoes a complex series of movements and tissue remodelling events that leads to the formation of the mature chambered organ⁸. Positional information for the formation of the atria and ventricles at specific sites within the heart tube, comes from the integration of the anterior-posterior, dorso-ventral and left-right patterning at early stages⁹. One of the first features of anterior-posterior patterning is the restriction of the ventricular myosin

heavy chain (VMHC1) and atrial myosin heavy chain (AMHC1) to the anterior and posterior pole, respectively, of the heart tube¹⁰⁻¹². In this study, we show that TH mRNA is enriched in the cardiac field of stage (st.) 5 chick embryos; by st. 8, TH expression was restricted to the splanchnic mesoderm of both endocardial tubes. Subsequently, TH localized predominantly to the myocardial layer of the posterior heart tube. Treatments with L-DOPA and dopamine induced ectopic expression of cardiac transcription factors and the contractile protein AMHC1, as well as sarcomere formation. Overexpression of TH led to the expansion of AMHC1 and Tbx5 expression into the anterior region, and induced bradycardia. Inhibition of dopamine biosynthesis or knockdown of TH decreased AMHC1 and Tbx5 expression, and perturbed correct cardiac looping. Exposure to retinoic acid (RA) induced and expanded TH expression, as well as that of AMHC1 and Tbx5, whereas blockage of endogenous RA synthesis inhibited TH expression. Our results show that TH is expressed in a dynamic pattern during the primitive heart tube formation. TH induces cardiac differentiation *in vivo* and it is a key regulator of the heart patterning, conferring atriogenic identity.

Methods

Experimental protocols with animals were performed in agreement with the Spanish law in application of the EU Guidelines for animal research, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23). Approval by the CIB bioethics board was obtained prior to the initiation of the study. For details on the following methods, see supplementary materials online.

Whole chick embryo culture and citral treatment.

Fertilized eggs (Granja Santa Isabel, Córdoba, Spain) were incubated at 38 °C in forced-draft, humidified incubators. Embryos were staged and cultured. For citral treatment, twenty microliters of test solution were applied over embryos at st. 5. Embryos were fixed in 4% PFA at st. 10-11 and processed for whole mount *in situ* hybridization (ISH).

Embryo electroporation.

St. 3 cultured embryos were injected and electroporated in the region determined to form the heart. For gain-of-function experiments the pCAGs- I-GFP (control) or pCAGs-TH-I-GFP vector was electroporated. For knock-down experiments, either fluorescein-labelled luciferase or TH morpholino oligonucleotide (MO) (Gene Tools LLC) was electroporated. After further 18-36 hour incubation, embryos were either fixed in 4% PFA and processed for whole mount ISH or the atria and ventricles were collected for RNA isolation.

Bead implantation.

Beads were soaked in the indicated solutions and implanted in the desired location at st. 5 (see Fig. 2A). After 6-18 hours of additional incubation, embryos were fixed in 4% PFA and processed for whole mount ISH or immunohistochemistry.

HPLC analysis.

St.8 and st.10 embryos were processed to detect catecholamines by HPLC with colorimetric detection.

ISH and Immunohistochemistry. Embryos were processed for ISH following standard procedures. The probes for cAMHC1, cVMHC1, Irx4, Bmp2 and cNkx2.5 have been described. The cTH and cTbx5 probes, were generated by RT-PCR cloning. Whole mount immunohistochemistry was performed using the MF20 antibody followed by anti-mouse Ig-HRP antibody.

RNA isolation and RT-qPCR.

Total RNA was isolated with the Trizol reagent and reverse transcribed with the Superscript III and random primers (Invitrogen). qPCR was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) by using TaqMan Universal PCR Master Mix, No-AmpErase UNG (Applied Biosystems) and the probes of the Universal Probe Library (URL, Roche Applied Science) were used for detection. Primer sequences and the respective URL probes are listed in supplementary material.

Plasmids.

The pCAGs-TH-I-GFP was generated by excising the TH cDNA from the pCRII-TOPO-TH, described above, with EcoRI and cloning it into the similarly digested pCAGs-I-GFP.

Results

Early TH expression is localized to the developing heart and is active prior to its appearance in the nervous system.

In postnatal organisms, TH is predominantly expressed in discrete areas of the brain, in the peripheral nervous system and in the adrenal gland. However, our identification of a dynamic pattern of TH expression in the early chick embryo¹³ led us to search for an as yet uncharacterized, preneuronal, function for TH. Whole mount ISH demonstrated that TH mRNA was expressed in st. 8 embryos, in the heart forming regions overlying the left and right endocardial tubes (Fig. 1B and S1). Transverse sections showed that TH mRNA was restricted to precardiac splanchnic mesoderm (Fig. 1Ba). At st. 9, TH mRNA was found specifically in the fusing cardiac tubes and, once the primitive heart tube was formed, TH displayed a graded pattern of expression. TH transcripts were concentrated in the posterior part of the looping heart, the prospective atrial region (Fig. 1B, st. 12). Indeed, no transcripts were evident at the anterior pole of the looping heart (the prospective ventricular region: Fig. 1Bb), whereas TH expression was restricted to the myocardial layer at the posterior pole (Fig. 1Bc). This pattern of gene expression is remarkably similar to that of other genes that become progressively restricted to the posterior heart tube [e.g. *AMHC1*¹⁰⁻¹² and *Tbx5*¹⁴⁻¹⁶, see figure S1 for better comparison]. Moreover, the distribution of TH transcripts coincided with that of TH protein (data not shown).

The selective cardiac expression of TH was confirmed by RT-qPCR and TH mRNA was detected in gastrulating st. 5 embryos, prior to the time specific staining can be observed by whole mount ISH. TH transcript levels were 2-fold higher in the heart field of st. 5 embryos than in the total embryo (Fig. 1C) and, overall, TH transcript levels rose as development continued (approximately 2-fold between st. 5 and 8, and 4-

fold between st. 8 and 10). This increase was more noticeable in the cardiac region, particularly between st. 5 and st. 8, in accordance with the distribution observed by ISH.

TH activity depends on allosteric factors and on the phosphorylation state of the enzyme, as well as on the presence of co-factors^{1,17}. To assess whether TH was active at these early embryonic stages, we measured catecholamine levels by HPLC in whole embryos at the endocardial, linear and looped tube stages (st. 8, 10 and 12, respectively). We chose not to dissect cardiac tissue to avoid material losses. L-DOPA was detected at st. 8 and their levels increased throughout cardiac development (Fig. 1D). Dopamine was detected only occasionally at st. 8, 10 and 12, and noradrenaline and adrenaline were not found at these embryonic stages. To explain the absence of catecholamines downstream of dopamine, we analyzed the expression of DBH. We could only detect DBH mRNA by RT-qPCR, and no specific signal by ISH was obtained. At all the stages analyzed, much lower levels of DBH mRNA than of TH mRNA were found (approximately 9, 80 and 190-fold lower at st. 5, 8 and 10, respectively). These differences were even greater (from 12 to 900-fold) in the cardiac region, indicating that the precursor L-DOPA and small amounts of dopamine are the principal, or perhaps the only, catecholamines present in the embryo prior to neuronal differentiation. The facts that dopamine is an unstable metabolite and the HPLC method is less sensitive for dopamine than for L-DOPA detection may account for the irregular detection of the former. These results extend previous findings, using less sensitive assays, where TH activity in the chick embryo was detected on the first day of incubation (st. 8), yet DBH activity was not evident until st. 19-20¹⁸.

L-DOPA and dopamine induce cardiac differentiation.

The pattern of TH expression and the presence of catecholamines suggested that TH and L-DOPA, and/or dopamine, play a role in cardiac development. We thus

implanted heparine-acrylamide beads soaked in either L-DOPA (10 $\mu\text{mol/L}$) or dopamine (10 $\mu\text{mol/L}$) lateral to one of the bilateral heart fields in embryos cultured at st. 5 (Fig. 2A, yellow bead). These embryos were allowed to develop until the linear or looped heart stage (st. 10 and 12, respectively), and were subsequently analyzed by whole mount ISH. St. 12 was the furthest developmental stage analyzed because the EC culture interferes with normal development beyond this stage. L-DOPA and dopamine induced expression of the cardiac transcription factors Nkx2.5 and Tbx5 in the ectopic tissue adjacent to the bead (Fig. 2A and Table S1). Moreover, expression of AMHC1, a sarcomeric protein and marker of terminal cardiac differentiation^{10,12}, was also induced, as seen by ISH and by immunohistochemistry with the MF20 antibody. The cells of the induced ectopic tissue adjacent to the bead developed myofibrils organized into sarcomeres, similar to those found in the cardiomyocytes of the primitive heart tube (Fig. 2B). No ectopic tissue or ectopic expression of cardiac genes was provoked by control beads (Fig. 2A), ruling out the possibility that implantation of the bead alone triggered ectopic cardiogenesis. This also argues against the possibility that the bead acts as a physical barrier for an inhibitory signal, and suggests that exogenous L-DOPA and dopamine can stimulate cardiomyocyte differentiation. Preliminary studies show that dopamine beads also induce the expression of Bmp2 (Fig. S2), linking TH to early cardiac differentiation programs¹⁹.

We further confirmed this hypothesis by blocking the endogenous synthesis of L-DOPA and dopamine. Accordingly, beads soaked in either 3-iodo-tyrosine (3I-Tyr, 1 mmol/L), an inhibitor of TH, or in meta-hydroxybenzylhydrazine (mHBH, 1 mmol/L), an inhibitor of L-DOPA decarboxylase (Fig. 1A), were implanted medial to one of the bilateral heart fields of cultured embryos at st. 5 (Fig. 2A, red bead). Blockage of L-DOPA, and more clearly of dopamine, biosynthesis led to an ipsilateral decrease of

AMHC1 expression in the endocardial tube at st. 8 (Fig. 2C and Table S2). These results suggest that dopamine synthesis is necessary for the correct expression of AMHC1.

Overexpression and knock-down of TH modified the limits of the cardiac chambers. To gain further insight into the role of TH in cardiac differentiation, we performed gain-of-function experiments. A bicistronic construct containing the chicken TH cDNA and the GFP cDNA (pCAGs-TH-I-GFP) was injected and electroporated, into the cardiac progenitor cells that were migrating through the primitive streak of st. 3 embryos. As a control, we used a vector containing the GFP cDNA alone (pCAGs-I-GFP). Analysis of TH expression in pCAGs-TH-I-GFP electroporated embryos confirmed that TH mRNA and protein coincided with GFP expression (Fig. S3). In addition, there was an increase in L-DOPA levels measured by HPLC in embryos overexpressing TH (data not shown). Consistent with the myocardiogenic stimulatory effect of the L-DOPA and dopamine beads (Fig. 2A), the embryos overexpressing TH displayed a marked increase in AMHC1 expression when compared with control electroporated or non-electroporated embryos, particularly at the stages of heart tube fusion and looping (st. 10 and 12, respectively; Fig. 3A). In addition, the domain of AMHC1 expression at the looped heart tube stage (st. 12) had expanded abnormally towards the anterior pole of the primitive heart tube (Fig. 3A). To further characterize the changes in the developing heart, we analyzed the expression of Tbx5. This T-box family transcription factor is expressed in cardiac progenitors and, as development proceeds, it is found in a graded fashion along the heart tube with the highest levels in the sino-atrial region¹⁴⁻¹⁶. Significantly, the pattern of Tbx5 expression in these tissues very closely resembles that of TH and AMHC1 (Fig. S1). Like AMHC1 expression, Tbx5 expression expanded abnormally towards the anterior region of the heart tube in

TH-overexpressing embryos (Fig 3A, Table S3). Moreover, RT-qPCR analysis of dissected ventricles showed a 6-fold increased of AMHC1 levels in TH overexpressing embryos when compared to control electroporated ventricles (Fig 3B). Tbx5 expression increased more modestly (not statistically significant) similarly to what was also observed by ISH.

We then analyzed the effect of TH on the expression of the ventricular myosin heavy chain, VMHC1, initially expressed throughout the entire heart tube and downregulated in the atria after the looping stage (st. 12-13)^{11,12,20}. VMHC1 became restricted to the anterior region of the heart tube of TH-overexpressing embryos (Fig. 3C, Table S4). Likewise, the expression of the transcription factor Irx4, which is involved in specifying the prospective ventricular region²⁰, regressed anteriorly (Fig 3C), suggesting that TH confers a posterior character on the looping heart tube.

Overexpression of TH also had major functional consequences, since TH-electroporated embryos displayed characteristics compatible with bradyarrhythmia (see movie online). When recorded in culture, the heart rate of control electroporated embryos at st. 11 was 100 beats per minute (bpm), compared to 53 bpm in TH-overexpressing embryos (Table 1). In addition, TH-electroporated embryos displayed arrhythmic heart beats (see movie online).

To further confirm that TH is a fundamental element in cardiac development, we knocked down TH expression. A TH MO or luciferase MO as a control, were electroporated into the cardiac progenitor cells migrating through the primitive streak of st. 3 chick embryos. The TH morphants displayed a decrease in the expression of the atrial markers AMHC1 and Tbx5 (Fig. 4A). Conversely, the expression of VMHC1 in the most affected embryos was increased (Fig. 4A). In parallel, the silencing of TH expression diminished the atrial segment area and oversized the ventricular segment

(Fig. 4B, Table S5). Similar anterior-posterior heart patterning disruption was observed when retinoic acid signaling was inhibited²¹. Additionally, the progress of cardiac morphogenesis was very limited: TH morphants displayed a globular cardiac tube that did not form a fully looped tube (Fig. 4B), and the dysmorphic hearts barely beat (data not shown).

TH action is linked to retinoic acid patterning effect.

To integrate these findings into what is already known about heart morphogenesis, we turned to the effect of retinoic acid (RA) known to be involved in cardiac patterning. In vertebrates, the anterior-posterior identity of the primitive heart tube is established by RA signaling²². The documented posteriorization of RA^{12,16,21,23,24} and that of TH described here, together with the fact that TH gene expression and activity are positively regulated by RA in the nervous system and adrenal gland^{25,26}, suggested that TH might be a putative downstream target of RA activity in establishing the anterior-posterior heart tube axis. We tested this hypothesis by implanting beads soaked in RA (10 µg/ml) into the heart field of cultured embryos at st. 5. RA expanded the expression of TH mainly in the ipsilateral inflow tract, as well as rostrally within the heart tube. A parallel effect was observed on the expression of AMHC1 and Tbx5 (Fig. 5A, Table S6). To further support that TH expression is under RA control, we inhibited endogenous RA synthesis with citral. Accordingly, cultured embryos treated at st. 5 with citral (10 mmol/L) displayed a dramatic decreased in TH expression parallel to diminished AMHC1 and Tbx5 expression (Fig. 5B, Table S7).

Discussion

The results presented here indicate that TH/L-DOPA/dopamine are involved in the network of signals that drive cardiac precursor cells to a sino-atrial fate, specifically by regionalizing Tbx5 and AMHC1 expression to the posterior part of the heart tube.

In st. 5 chick embryos, TH mRNA was enriched in the cardiac field; by st. 8, TH expression localized to the splanchnic mesoderm of endocardial tubes and it progressively became restricted to the sino-atrial region. Perturbing graded TH expression altered atrial and ventricular myosin segregation. Thus, while an increase in TH levels resulted in expansion of the AMHC1 and Tbx5 atrial domains and regression of the VMHC1 and Irx4 ventricular domains, loss of TH activity decreased AMHC1 and Tbx5 expression, and occasionally increased VMHC1 expression. The less widespread VMHC1 expansion phenotype indicates that other factors are probably involved in restricting VMHC1 expression. Significantly, it has been shown that the graded expression of Tbx5 is crucial for the correct cranio-caudal patterning of the primitive heart tube, since disruption of this pattern results in abnormalities in heart chamber formation¹⁴⁻¹⁶. Indeed, the Holt-Oram syndrome in humans (with structural cardiac and conduction anomalies) is associated with mutations in the *TBX5* gene²⁷⁻²⁹. The graded expression of Tbx5 and the posterior identity of the primitive heart tube are maintained by RA. An excess of RA causes expansion towards the anterior region of genes that are normally concentrated to the posterior region (i.e. Tbx5 and AMHC1), together with hyperplasia of the sino-atrial region^{12,16,21,24}. Conversely, inhibition of RA signaling impairs development of the sino-atrial region and enlarges that of the ventricular region^{21,23}. In our model system, we uncover a link between RA and TH action as suggested by the parallelism in the effect of blockage of endogenous TH expression and inhibition of RA signaling²¹, together with the fact that RA controlled TH expression. Thus, according to these results, TH appears to reinforce the genetic program of the sino-atrial region, in a scenario where RA modulates the graded TH expression in the heart tube. In concert, TH activity favors the restriction of Tbx5 and

AMHC1 expression to the posterior heart tube, while suppressing *Irx4* and VMHC1 in the prospective atria (Fig 5C).

Alteration of TH expression (gain or loss) had also important functional consequences. Increased TH expression led to low rate and arrhythmic heart beating while loss of TH activity caused partial looping and discontinuous beating of the heart tube. The cause of this abnormal functionality is an open question. Previous studies have shown that restricting expression of contractile proteins to the ventricular and atrial chamber, including that of the myosin heavy and light chains, is essential for normal cardiac function³⁰⁻³². Given that TH overexpression alters the distribution of atrial and ventricular myosin heavy chain isoforms, this could be at least partially responsible for some of the heart function abnormalities found in the TH-overexpressing embryos. Nevertheless, we are tempted to speculate that TH might be involved in specifying the cardiac pacemaker of the embryonic heart. In the chick, the pacemaker differentiates at around st. 9-10 in the posterior most segment of the primitive heart tube³³, assuring the rhythmic propagation of the action potential with posterior-anterior polarity. The bradyarrhythmia of the TH-overexpressing embryos, together with the restriction of TH expression to the sino-atrial region, are compatible with the interpretation that the gradient of TH activity in the primitive heart tube may be part of the signals leading to the dominance of the pacemaker in the posterior tube. This phenomenon could also be responsible for the development of the prospective sino-atrial node responsible for the pacemaker activity in the adult heart. This hypothesis is consistent with Pollack's proposal several decades ago³⁴ (reviewed by Ebert³⁵) that catecholamines are essential for cardiac pacemaker specification. Future experiments analyzing the expression of early pacemaker genes, among others, should clarify the role of TH in the pacemaker specification.

Generation of the catecholamine depleted mice models (TH and DBH knockouts)³⁻⁵ have more recently shown that catecholamines are essential for embryo survival beyond mid-gestation. The catecholamine deficient mice die from apparent heart failure starting at E11.5-12.5, and the heart of surviving embryos is able to beat autonomously with slight bradycardia. Albeit the penetrance of the lethal phenotype was variable and some null-mice survived to term, recent reports have shown that catecholamines, in particular noradrenaline, mediate fetal survival by maintaining oxygen homeostasis in mid-gestation^{6,7}. Moreover, restoration of noradrenaline, although not of dopamine, synthesis in the noradrenergic cells is sufficient to prevent lethality³⁶. Using the chick embryo as a maternally-independent vertebrate model, we show that catecholamines, in particular dopamine, also have an earlier role in cardiac morphogenesis. Similar to our results in the chick embryo, in the study by Thomas *et al.*⁴ dopamine was the only catecholamine detected in E9.5 mouse embryos (long before the lethality occurred). In fact, we could detect TH expression in the whole mouse embryo since gastrulation stage at E6.5 and in the heart since E8.5 (the earliest stages analyzed) (Fig. S3). Our results are compatible with the findings in mice: dopamine plays an important role in early cardiac tube formation, whereas noradrenaline can be essential for mid-gestation fetal survival. Moreover, the apparent absence of major histological alterations in the catecholamine null-mice could be due to the presence of compensatory factors, including a potential contribution of maternal catecholamines⁴ or to species variation. A detailed characterization of the phenotype in mouse organogenesis would be required to unravel the role of all catecholamines in mouse cardiogenesis.

Here, we have shown a strong concordant phenotype in TH-gain and loss-of-function experiments in chick embryos. The remarkable change in the pattern of

electrical activation in the TH-overexpressing hearts is also consistent with the identification of intrinsic catecholamine-synthesizing cardiac cells, in human and rodent hearts, prior to innervation^{37,38}. In mice and rats, cardiac catecholaminergic cells appear to be associated with the pacemaker and conduction system in four-chamber hearts³⁷.

The three dimensional requirements of cardiac development need complex signalling networks to lead cells through the appropriate fate decisions and morphogenetic movements^{8,39}. In addition, antero-posterior polarity plays a role in the coupling between heart and blood vessels. Here, we reveal a novel function of TH in cardiac development, and suggest that TH may be a key player acting in concert with additional factors to define multiple aspects of chamber identity, including pacemaker specification. These results are consistent with the paradigm whereby molecules that act as intercellular signaling mediators, with well-defined restricted roles in postnatal organisms, are present at embryonic stages, when they participate in diverse functions often unrelated to their later roles (reviewed in^{40,41}). The relevance of TH in human cardiogenesis and the possible significance of TH pathway alterations in cardiac syndromes may be a field deserving further studies.

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Figure Legends

Figure 1. Tyrosine hydroxylase expression and activity in developing chick embryos. A. The catecholamine biosynthesis pathway. B. Whole-mount ISH for TH at stages 8-12. a, b and c correspond to transverse paraffin sections at the levels indicated by the corresponding lines. Note that at st. 8 TH mRNA is restricted to the splanchnic mesoderm of the endocardial tubes, and later it is predominantly expressed in the myocardial layer of the atrigenic region (arrows in a and c). C. RT-qPCR of RNA from whole embryos at stages 5, 8 and 10 or from their corresponding cardiac regions. The levels of TH mRNA were normalized to GAPDH mRNA levels. The results represent the mean \pm SD of three experiments. D. L-DOPA was measured by HPLC in extracts from one pool of 30 st. 8 embryos, two pools of 15 st. 10 embryos and two pools of 10 st.12 embryos. The results represent, except in st. 8, the mean \pm SD.

Figure 2. Effect of L-DOPA and dopamine, and their inhibition, on cardiac development. A. Effect of L-DOPA and dopamine on the expression of cardiac genes. Left drawing corresponds to a st. 5 embryo with a bead implanted lateral to one of the bilateral heart fields (yellow bead). Beads were soaked in either PBS (vehicle), or a solution of 10 μ mol/L L-DOPA or dopamine. Stage 10-12 embryos were subjected to whole-mount ISH for Nkx2.5, Tbx5 and AMHC1, or immunohistochemistry for MF20. Ectopic tissue adjacent to the bead coated with L-DOPA or dopamine (arrow) expressed all markers. Labelling was not detected around the control bead (arrowhead). B. Ultrastructure of the ectopic tissue induced by dopamine. Semi-thin sections of the bead area and ectopic tissue (a: 40x). Transmission electron microscopy of cells adjacent to the dopamine bead (b: 20,000x) or of cardiomyocytes in the primitive heart tube (c: 10,000x). The white arrowheads indicate the Z bands, and the purple and yellow lines delineate the I and A bands, respectively. C. Effect of the inhibition of L-DOPA or

dopamine synthesis on AMHC1 expression. The position of the bead implanted medial to one of the bilateral heart fields of a st. 5 embryo is shown in 2A (red bead). Beads were soaked in either 3I-Tyr or mHBH, or the vehicle solution (NaOH and PBS, respectively). Embryos at st. 8 were analysed by whole-mount ISH for AMHC1. Note that AMHC1 expression is inhibited in the endocardial tube ipsilateral to the bead (arrow), but not by the control bead (arrowhead).

Figure 3. Effect of TH on anterior-posterior heart tube patterning and specification of chambers. A. Effect of TH gain-of-function on sino-atrial gene expression. Whole-mount ISH for AMHC1 and Tbx5 in non-electroporated embryos, and in embryos electroporated with either the control construct (pCAGs-I-GFP) or with the TH expressing construct (pCAGs-TH-I-GFP). The anterior expanded expression of AMHC1 and Tbx5 in the heart tube is indicated by the black arrows. Visualization of GFP expression for the embryos processed for ISH is shown in the corresponding left panels. B. RT-qPCR of RNA from ventricles (V in scheme) of st. 12 embryos electroporated with either the control construct (pCAGs-I-GFP) or with the TH expressing construct (pCAGs-TH-I-GFP). The levels of AMHC1 and Tbx5 mRNA were normalized to GAPDH mRNA levels. The results represent the mean \pm SEM of three pools of 4 ventricles each. *P < 0.01 with respect to pCAGs-I-GFP electroporated embryos. C. Effect of TH gain-of-function on ventricular gene expression. Whole-mount ISH for VMHC1 and Irx4 in embryos as in (A). The posterior regression of VMHC and Irx4 expression is indicated by the red arrows. In supplementary materials this figure is reproduced including GFP expression (Fig. S4).

Figure 4. Effect of TH knockdown on anterior-posterior heart tube patterning and specification of chambers. A. Whole-mount ISH for AMHC1, Tbx5 and VMHC1 in embryos electroporated with either luciferase MO or with TH MO.

Note also the decrease in AMHC1 and Tbx5 expression in the TH MO treated (arrow) versus de luciferase MO control (arrowhead). On the contrary, VMHC1 expression did not change or showed a mild increase. B. Light microscopy of st. 10 and 12 non-electroporated or electroporated embryos with either luciferase MO or with TH MO. The heart tube of the TH morphants show abnormal morphogenesis displaying an atrophic sino-atrial region and oversized ventricular region (in embryos with comparable number of somites and similar prosencephalon development). The ventricular segment is outlined in blue and the sino-atrial segment in yellow.

Figure 5. TH, AMHC1 and Tbx5 expression are controlled by retinoic acid.

A. Increased TH, AMHC1 and Tbx5 expression by retinoic acid. St. 5 embryos with a bead soaked in either DMSO (vehicle) or RA implanted lateral to one of the bilateral heart fields (yellow bead) were then analyzed by whole-mount ISH at st. 12. The expanded expression of TH, AMHC1 and Tbx5 in the heart tube and in the inflow tract are indicated by arrows, and their normal expression domains are outlined by yellow dotted-lines. B. Decreased TH, AMHC1 and Tbx5 expression by retinoic acid synthesis inhibition. St. 5 embryos were treated with ethanol (vehicle) or 10 mmol/L citral and analyzed by ISH at st. 10-11. C. Scheme of the interplay of RA and TH in chamber-specific genes regulation.

Table 1. Effect of TH overexpression on heart rate

	Non-electroporated (n=10)	pCAGs-I-GFP (n=10)	pCAGs-TH-I-GFP (n=10)
BPM*	100.6 +/- 3.53	100.5 +/- 3.02	53.1 +/- 2.68

*BMP: Beats per minute
The results represent the mean ± SD

Figure(s)

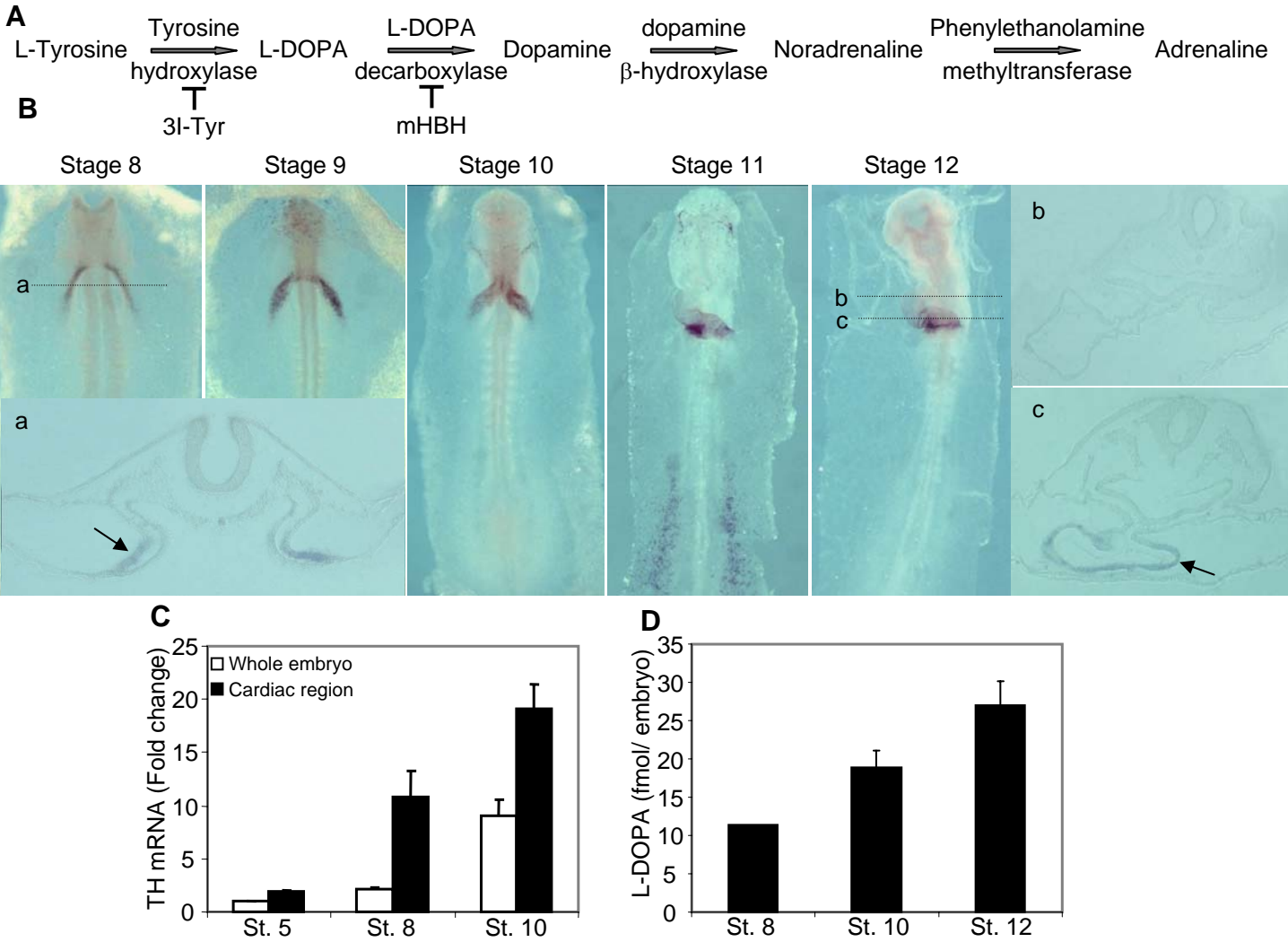


Figure 1

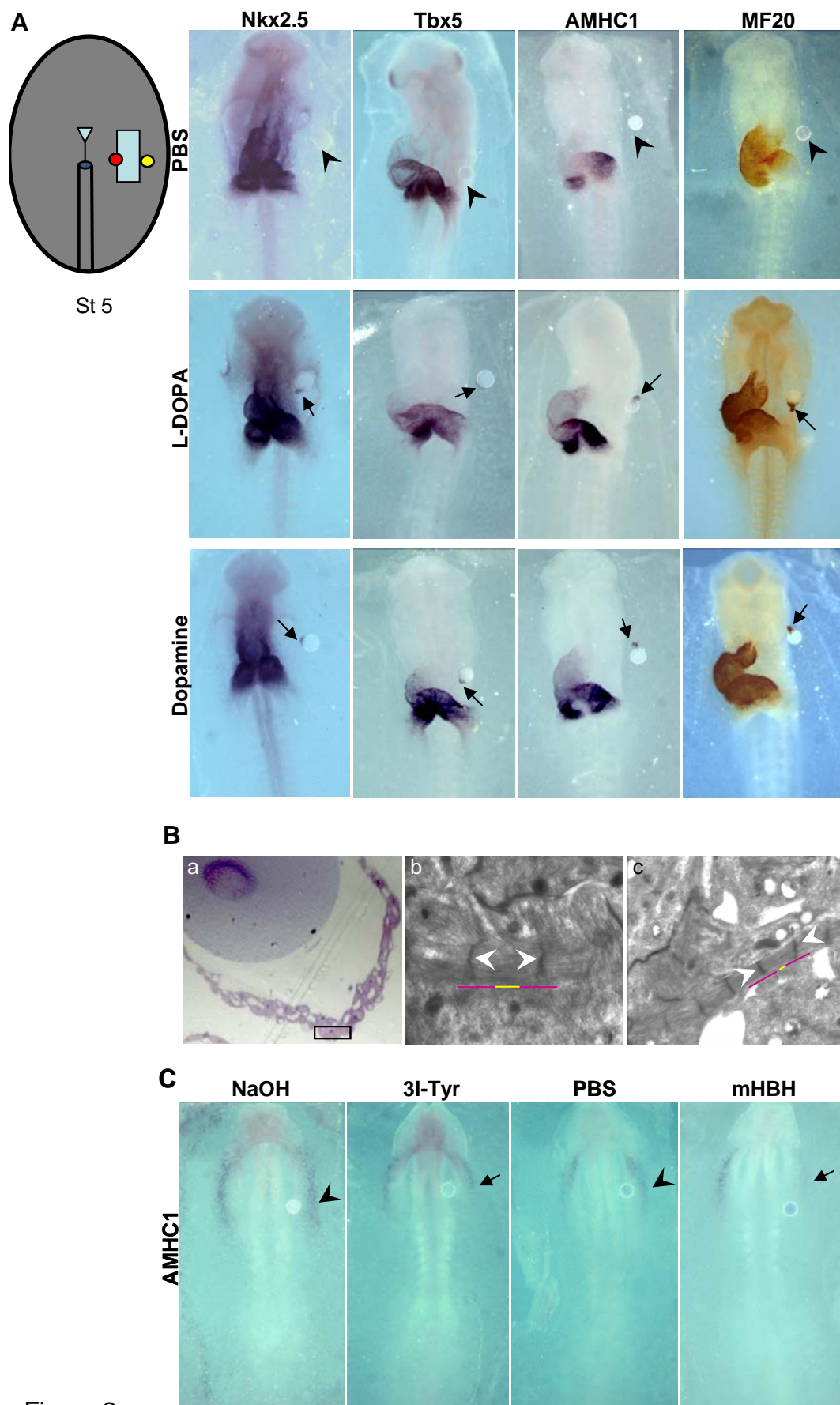


Figure 2

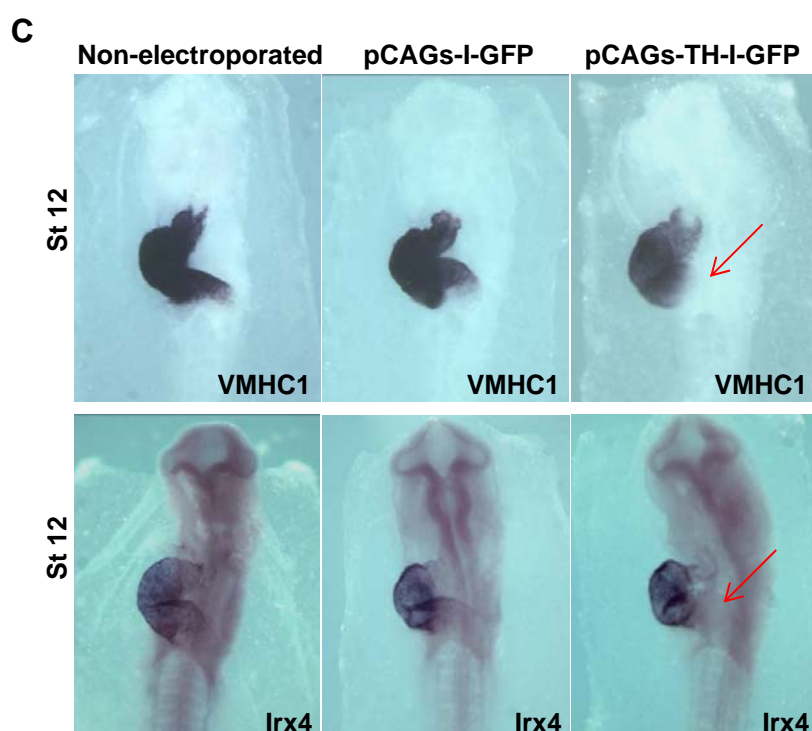
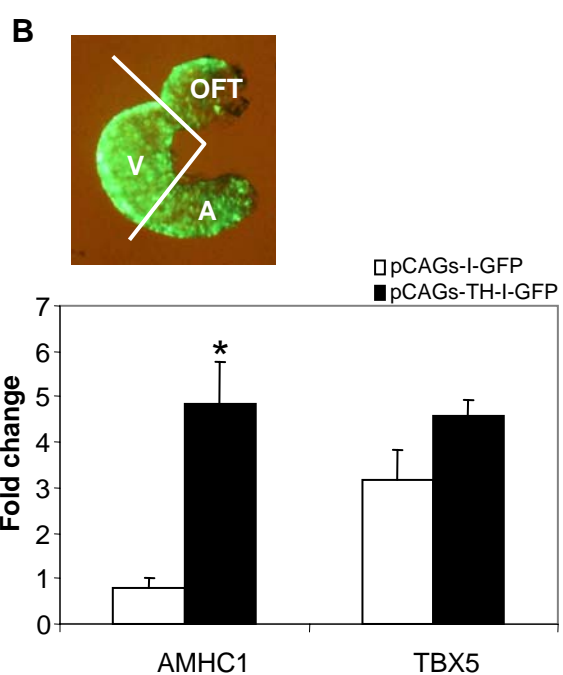
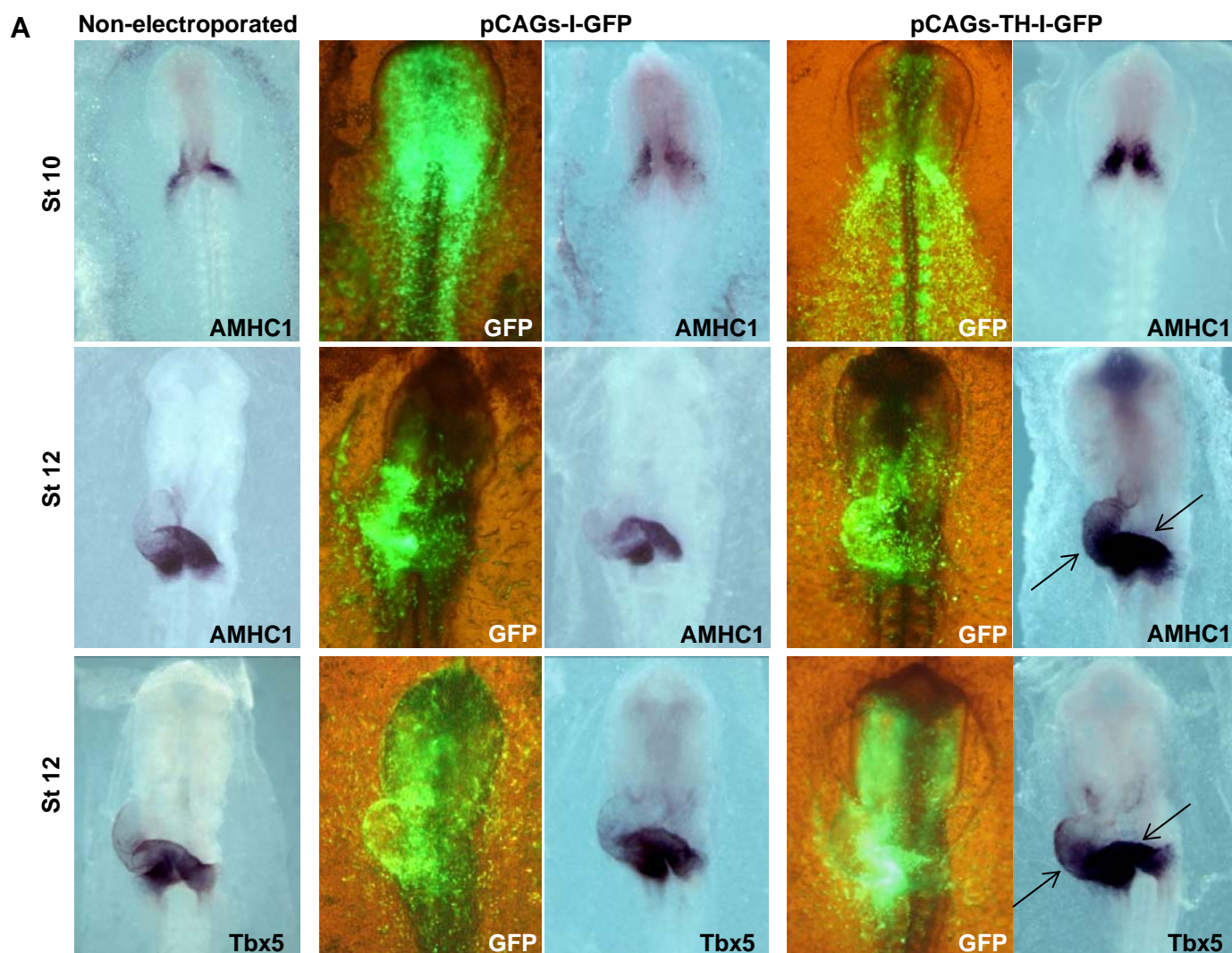


Figure 3

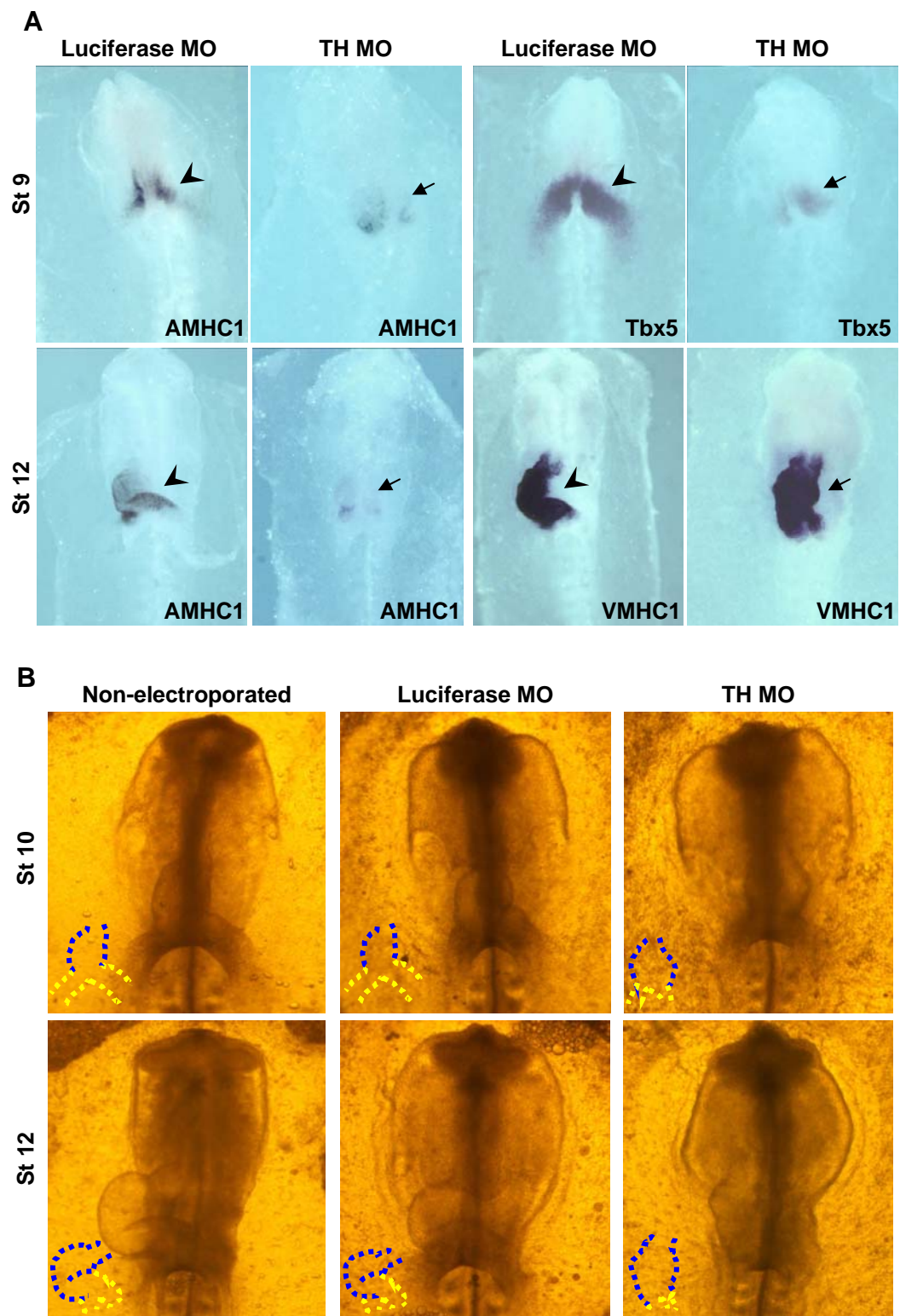


Figure 4

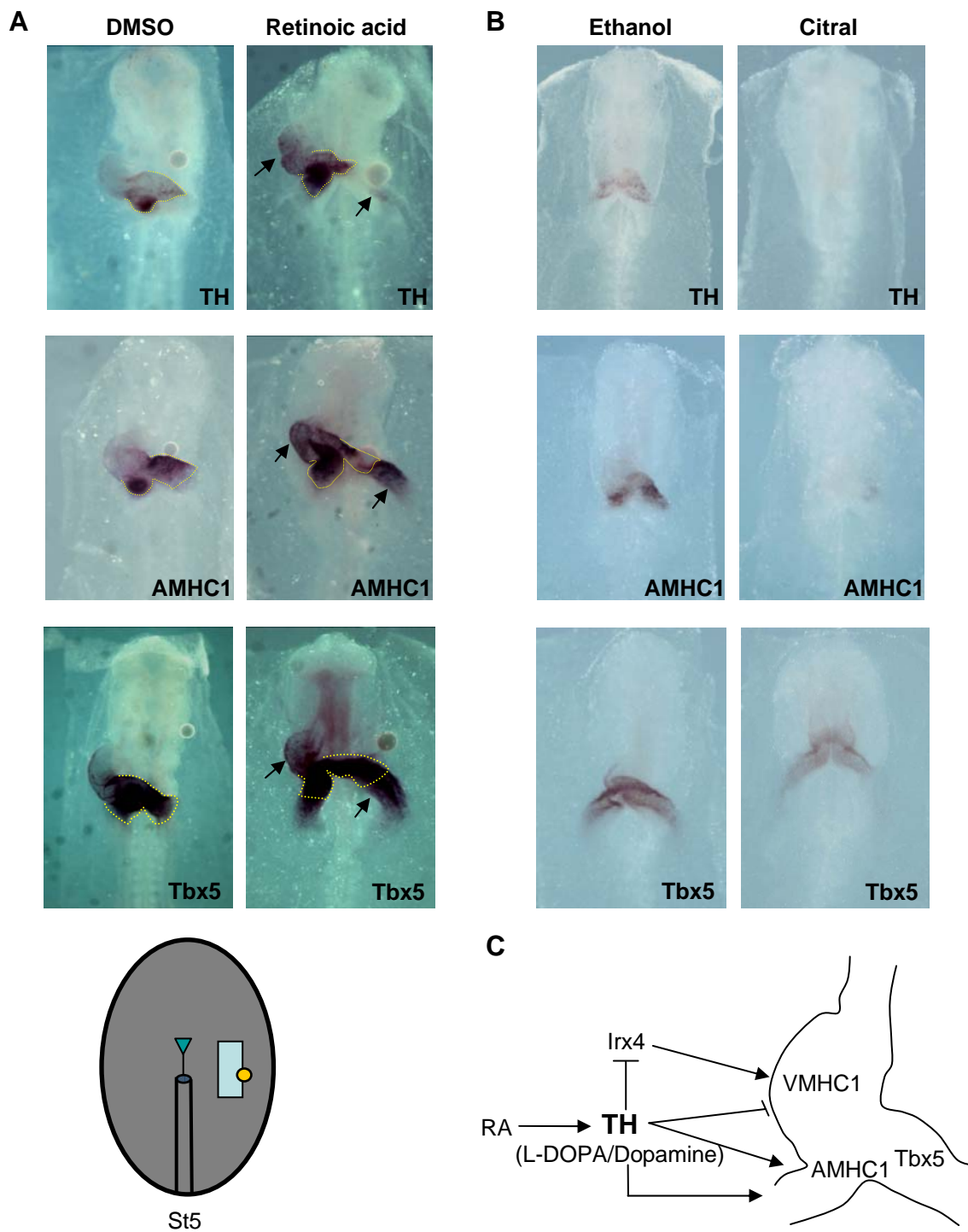


Figure 5

CVR-2010-9R1**Supplementary Methods****Whole Embryo culture.**

Embryos were staged according to Hamburger and Hamilton (1). The early chick (EC) embryo culture is an improved variant of the original New culture (2) as described by Chapman et al. (3). In essence, the egg is open and the albumen discarded. The yolk containing the embryo of the desired stage (st.) is laid on a Petri dish, cleaning with tissue the remaining albumen. A filter paper (20x20 mm Whatman) cut in the center is placed around the embryonic area, and the surrounding viteline membrane is cut out. The embryo containing filter piece is carefully lifted and placed ventral-side-up on a 35 mm Petri dish half-full with semisolid culture medium. This medium is 50% 123 mM NaCl / 0.6% agar and 50% albumen from non-incubated eggs.

Bead implantation.

L-DOPA, Dopamine and meta-hydroxybenzylhydrazine (mHBH) were dissolved in PBS, 3-Iodo-Tyrosine (3I-Tyr) was dissolved in 1 N NaOH, and all-*trans*-retinoic acid (RA) was dissolved in dimethyl sulphoxide (DMSO), (all from Sigma, St. Louis, MO). For L-DOPA, Dopamine, 3I-Tyr and mHBH treatments, heparin-coated acrylic beads (150-200 µm diameter, Sigma) were used, whereas for RA, AG1-X2 ion-exchange resin beads (150-200µm diameter, BioRad) were used. Heparin-coated acrylic beads were washed in phosphate-buffered saline (PBS) 3 times for 10 min each and then placed in a Petri dish containing a drop of the testing solution. After 2 h of incubation, individual beads were picked up with tweezers and implanted into a pocket opened between the epiblast and endoderm of cultured embryos at st. 5, in the desired location: medial or lateral to one of the bilateral heart field, as previously described (4). AG1-X2 ion-exchange resin beads were first soaked in 10 µg/ml RA for 20 min at room temperature, followed by staining in 0.05% Neutral Red for 10 min, and rinsed three times in 0.9% saline solution. Beads were placed in the embryo as described above. Control beads were soaked in PBS for experiments with L-DOPA, dopamine and mHBH treatments, in 1 mM NaOH for experiments with 3I-Tyr, and in DMSO for RA experiments. After 6-18 hours of additional incubation, embryos were fixed overnight in 4% PFA and processed for whole mount ISH or immunohistochemistry.

Citral treatment.

Cultured embryos at st. 5 were treated with citral (3,7-dimethyl-2,6-octadienal, Sigma), an inhibitor of retinoic acid synthesis (5). Stock solution of citral (1 M in

ethanol 100%) was diluted in PBS to 10 mM. Control embryos received ethanol 1% in PBS. Twenty microliters of test solution were applied over the embryo as described by Hochgreb et al. (6). Embryos were photographed at st. 10-11 under bright light, fixed overnight in 4% PFA, and processed for whole mount ISH.

Electroporation of precardiac primitive streak cells.

For electroporation we used a modified technique previously described by Colas and Schoenwolf (7). Cultured st. 3 or st. 3+ embryos were transferred ventral-side-up to a Petri dish containing the tungsten cathode electrode (tip 2 mm long and 100 μ m diameter, CUY701P2E, Nepagene) embedded in the agarose. The primitive streak of the embryo was aligned with the cathode located underneath. Next, a plasmid or oligonucleotide DNA solution was microinjected using an Inject + Matic microinjector system (INJECT + MATIC, Geneva) into the desired region of the primitive streak. Immediately after DNA injection, the anode (L-shape platinum, tip 2 mm long and 300 μ m diameter, CUY613P2, Nepagene) was placed just over the injected primitive streak area, and electric pulses were applied. A train of five square wave pulses (40-ms duration at 5V with 999-ms intervals) was applied using a square wave TSSS20 Ovodyne Electroporator (Intracel).

The mixture for plasmid or oligonucleotide DNA injection, was prepared immediately before electroporation. For plasmid (pCAGs- I-GFP or pCAGs-TH-I-GFP vector) a working solution that contained the plasmid to a final concentration of 1.5 μ g/ μ l, 1/10 volume of 60% (wt/vol) sucrose and 1/10 volume of 0.5% (wt/vol) fast green FCF (Sigma). For morpholino oligonucleotide (fluorescein-labelled luciferase or TH morpholino, Gene Tools LLC), a working solution that contained the morpholino to a final concentration of 1 mM, 0.3 μ g/ μ l pBluescript plasmid DNA as a carrier, 1/10 volume of 60% (wt/vol) sucrose, 1/10 volume of 0.5% (wt/vol) fast green FCF was prepared.

After electroporation, the embryos were transferred to the agar-albumen dish, returned to the incubator, and allowed to develop until they reached the desired stage. All the embryos were photographed under bright and fluorescent light (Nikon digital, SIGHT DS-U1). Embryos were selected according to the location and extent of the electroporation. The selected embryos were fixed in 4% PFA and processed for analysis of gene expression (ISH) and/or immunochemistry. A group of embryos electroporated were selected for RNA isolation; the atria and ventricles were collected and directly

frozen on dry ice for RT-qPCR analysis. In each experiment, *n* refers to the number of embryos successfully labelled and studied.

HPLC analysis.

St.8 and st.10 embryos were collected in 100 µl 0.3 N HClO₄ containing 0.4 mM sodium bisulphite and 0.4 mM EDTA. DHBA (100 pmol/ml) was added as an internal standard. Samples were sonicated and centrifuged at 15,000 *g* for 5 min at 4°C. The catecholamine content in 40 µl of the supernatant fraction was quantified by high-performance liquid chromatography (HPLC) with colorimetric detection, as described (8).

Whole-Mount *In situ* Hybridization (ISH).

In situ hybridization was performed using as probes the chick AMHC1 (9), VMHC1 (10), cNkx2.5 (11), Irx4 (12) and Bmp2 (13). All these probes were kindly provided by others labs. The cTH probe (1570-bp), and cTbx5 probe (1577-bp) were generated by reverse transcription (RT) from st. 10 total embryo RNA using the Invitrogen Superscript III kit and an oligo-dT primer, followed by PCR with the specific primers listed below. The amplified products were cloned into the pCRII TOPO shuttle vector (Invitrogen), and their identity and orientation were confirmed by sequencing.

Selected embryos were fixed overnight in 4% PFA, dehydrated in a graded methanol series in PBT (PBS, 0.1% Tween 20) and stored in 100% methanol at -20°C until further processing. For the probe hybridization step, embryos were rehydrated and permeabilized three times for 20 min in detergent solution (1% IGEPAL, 1% SDS, 0.5% deoxycholate, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 150 mM NaCl) then fixed for 20 min in 4% PFA. After several washes in PBT to remove the fixative, the embryos were incubated in prehybridization solution (50% formamide, 5x SSC, pH 4.5, 2% SDS, 2% BBR, 250 µg /ml tRNA, 100 µg/ml Heparin) at 65°C for 2 h followed by the addition of 10 µg/ml of the appropriate probe and further overnight incubation at the selected hybridization temperature. Then embryos were washed twice for 30 min with solution X (50% formamide, 2xSSC, pH 4.5, 1% SDS), at the hybridization temperature followed by two washes for 30 min. at room temperature in MABT (150 mM NaCl, 100 mM Maleic acid, 0.1% Tween 20). For the antibody step, nonspecific binding was blocked by incubating 1 h in blocking solution (MABT containing 2% blocking reagent, Roche). Embryos were incubated with AP-conjugated anti-DIG antibody (1:2000 in blocking solution, Roche) overnight at 4°C with rocking. The embryos were washed in MABT six times for 30 min at room temperature, followed by

two washes for 10 min in NTMT (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂, 0.1% Tween 20). The AP conjugated anti-DIG antibody was detected by a mixture of 4.5 µl NBT/3.75 µl BCIP (Roche) in 1 ml NTMT, pH 9.5. The reaction was stopped by washing in PBT once the required staining intensity was achieved, then embryos were fixed in 4% PFA overnight and photographed.

Embryos for histology were dehydrated with an ethanol series and then cleared in isopropanol and processed for paraffin embedding. Serial transverse sections were cut at 15 µm.

Immunohistochemistry.

Whole mount immunohistochemistry was performed as previously described by us (14), using the MF20 antibody [1/100, Hybridoma Bank, Developmental Studies (15)] followed by anti-mouse Ig-HRP antibody (1/200, Jackson ImmunoResearch Laboratories, West Grove, PA), or the polyclonal rabbit anti-TH serum (1/100, Chemicon) followed by anti-rabbit Ig-HRP antibody (1/1,000 Chemicon). To obtain a brown reaction product, we performed a standard peroxidase reaction according to the manufacturer's instructions (solution obtained from diaminobenzidine [DAB] tablets, Sigma). When indicated, whole mount immunohistochemistry was performed after ISH.

Videotaping.

Control- and TH-electroporated groups of embryos in EC culture were incubated in a humidified chamber with a video recording system, at 38 °C for 60 min. At the end of this period, the embryos were recorded for 5 min and heart beats were counted for this time period.

Mouse embryos.

C57BL/6 mouse embryos were removed from the uterus of pregnant females and subsequently dissected from the deciduum. All animals were handled according to European Union Guidelines for animal research.

RNA isolation and standard and quantitative RT-PCR.

Total RNA from whole embryos or dissected heart regions was isolated with the Trizol reagent (Invitrogen) and 2.5 µg of RNA was typically reverse transcribed (RT) with the Superscript III Kit and random primers (all from Invitrogen). Quantitative PCR (qPCR) was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) by using TaqMan Universal PCR Master Mix, No-AmpErase UNG (Applied Biosystems) and the probes of the Universal Probe

Library (URL, Roche Applied Science) were used for detection. Chick (c) and mouse (m) primer sequences and the respective URL probes are listed below.

Primers and URL probes for qRT-PCR

Gene	Primers	URL
cTH	ACAGCCCCCAGACCATCT (left) AATCAGCGAATGAAGCTCGT (right)	80
cGAPDH	GTCCTCTCTGGCAAAGTCCA (left) ACCATGTAGTTCAGATCGATGAAG (right)	49
cDBH	TACAAGGTGTCCCTCGATCC (left) GCTCGGGGTAAGTACGTT (right)	66
cAMHC1	CTTTGTCCGCTGTCTCATCC (left) AGGGGGTTGTCCATCACAC (right)	12
cTbx5	GATGAGAACAACGGCTTTGG (left) AGTCTCGGGGAAGACATGG (right)	8
mTH	GGAACGGTACTGTGGCTACC (left) CCTTCAAGAAGTGAGACACATCC (right)	56
m18S rRNA	TGCGAGTACTCAACACCAACA (left) TTCCTCAACACCACATGAGC (right)	70

Primers for standard RT-PCR

Gene	Primers
cTH	CACGAGTGAAGATGCCAAC (left) CCCTGTTCCCTAGTCTGTAAC (right)
cTBX5	AAGCTCGTAACATGGCGGAC (left) TTAGCTGTTCTCGCTCCACT (right)
mTH	TCAGTGATGCCAAGGACAAG (left) ATCAAAGGGTCCAGCCAC (right)

TH morpholino oligonucleotide

GGGTTGGCATCTTCACTCTTCCACG

Supplementary tables

Table S1. Ectopic expression of cardiac markers induced by L-DOPA and dopamine

Heparin bead soaked in	cNkx2.5 (n)	Tbx5 (n)	AMHC1 (n)	MF20 (n)	Total (n)
PBS (control)	0/9	0/10	0/12	0/15	46
L-DOPA (10 μ M)	8/8	9/9	15/15	12/12	44
Dopamine (10 μ M)	9/9	9/9	16/16	14/14	48

Table S2. Inhibition of AMHC-1 expression

Heparin bead soaked in	Decreased AMHC1 expression (n)
NaOH (control)	0/12
3I-Tyr (1 mM)	16/16
PBS (control)	0/10
mHBH (1mM)	15/15

Table S3. Effect of TH overexpression on atrial markers

Electroporated vector	Expanded AMHC1 expression (n)	Expanded Tbx5 expression (n)
pCAGs-I-GFP	0/21	0/15
pCAGs-TH-I-GFP	27/27	18/18

Table S4. Effect of TH overexpression on ventricular markers

Electroporated vector	Decreased VMHC1 expression (n)	Decreased Irx4 expression (n)
pCAGs-I-GFP	0/8	0/11
pCAGs-TH-I-GFP	12/12	15/15

Table S5. Effect of TH knockdown on anterior-posterior patterning

MO applied	Decreased AMHC1 expression (n)	Decreased Tbx5 expression (n)	Decreased VMHC1 expression (n)
Luciferase MO	0/12	0/8	0/9
TH MO	18/18	9/9	0/11

Table S6. Number of embryos affected by retinoic acid treatment

AG1-X2 bead soaked in	Expanded TH expression (n)	Expanded AMHC1 expression (n)	Expanded Tbx5 expression (n)
DMSO (control)	0/12	0/10	0/6
RA (10 µg/ml)	18/18	14/14	9/9

Table S7. Number of embryos affected by citral treatment

Treatment	Decreased TH expression (n)	Decreased AMHC1 expression (n)	Decreased Tbx5 expression (n)
Ethanol (control)	0/5	0/5	0/4
Citral (10 mmol/L)	12/12	9/9	6/6

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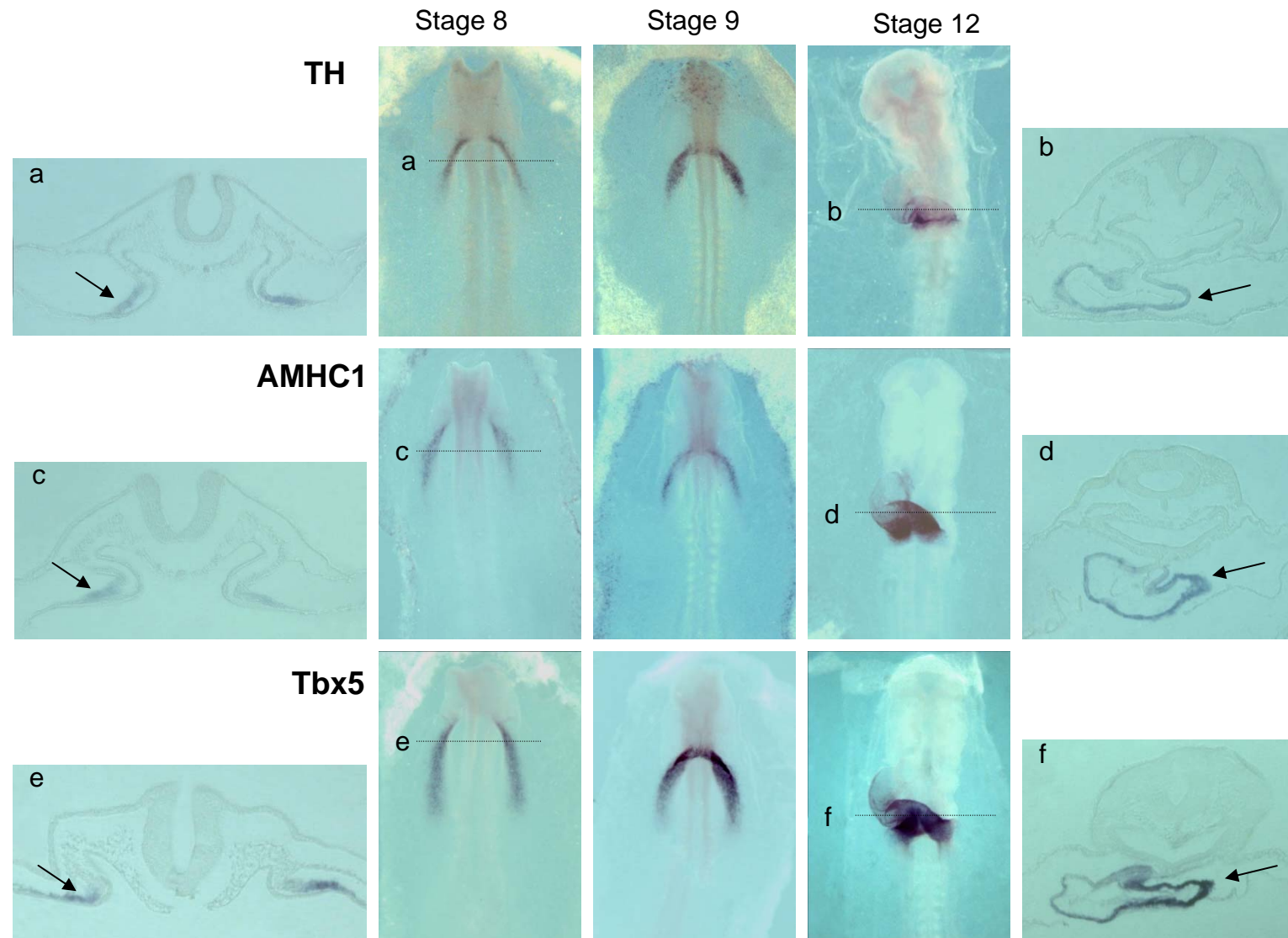


Figure S1. TH expression parallels that of AMHC1 and Tbx5 during cardiac tube formation. Whole-mount ISH for TH, AMHC1 and Tbx5. a-f correspond to transverse paraffin sections at the levels indicated by the lines. Note that at st. 8 TH mRNA as well as that of AMHC1 and Tbx5 are restricted to the splanchnic mesoderm of the endocardial tubes (a, c and e), and later it is predominantly expressed in the myocardial layer of the atrigenic region. The three genes are widely expressed in the endocardial tubes (st. 8) and in the fusing cardiac primordia (st. 9). Later (st. 12), the three mRNAs concentrate at the posterior part of the primitive heart tube, the prospective atrial region (b, d and f).

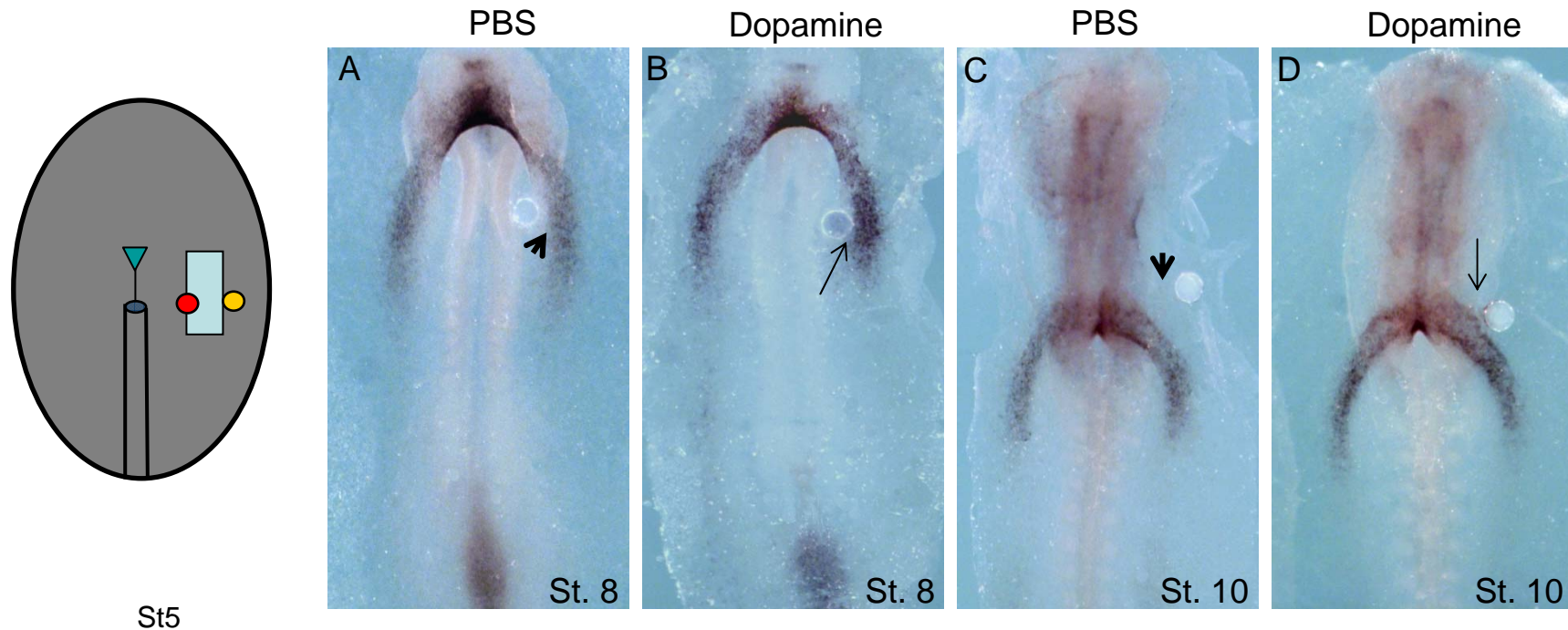


Figure S2. Effect of dopamine on BMP2 expression. Left drawing corresponds to a st. 5 embryo with a bead implanted lateral (yellow bead) or medial (red bead) to one of the bilateral heart fields. Beads were soaked in either PBS (vehicle), or a solution of 10 $\mu\text{mol/L}$ dopamine. Stage 8 (A-B) or st. 10 (C-D) embryos were subjected to whole-mount ISH for BMP2. A, B. Dopamine beads implanted medial to the heart field increased the expression of BMP2 in the endocardial tube ipsilateral to the bead (arrow), but not the control bead (arrowhead). C, D. Ectopic tissue adjacent to the bead coated with dopamine and implanted medial to the heart field expressed BMP2 (arrow). Ectopic tissue or labelling was not detected around the control bead (arrowhead).

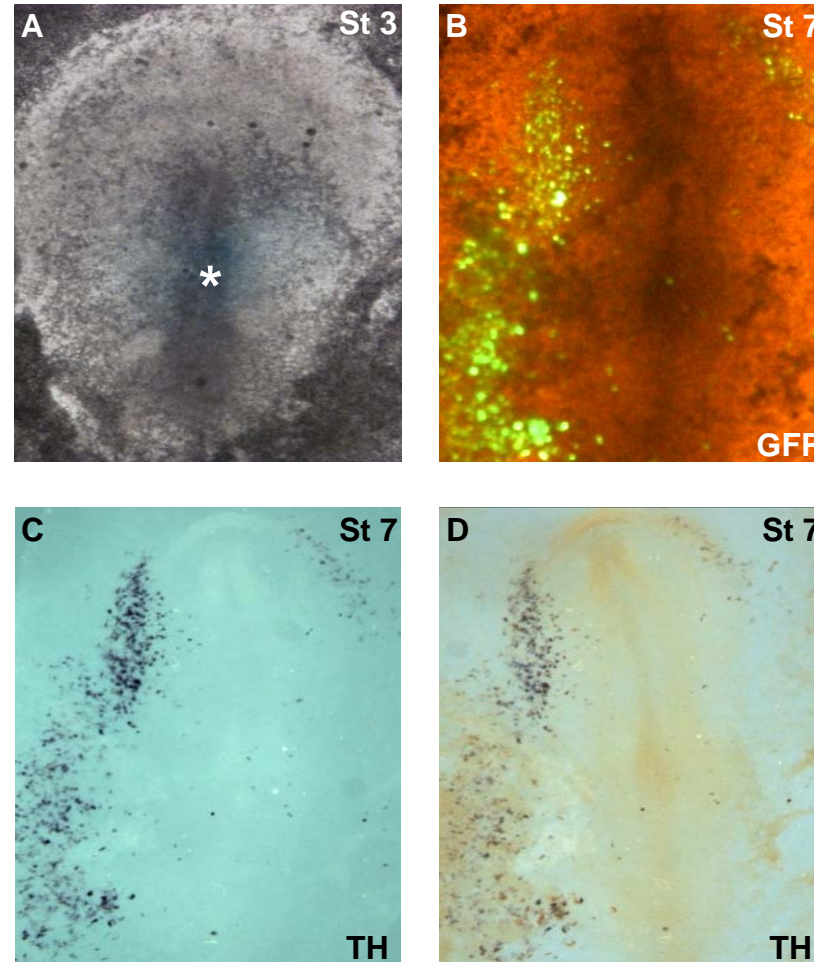


Figure S3. Co-expression of TH and the reporter gene in electroporated embryos. A. Embryo at st.3 injected and electroporated in the primitive streak (asterisk) with pCAGs-TH-I-GFP construct. B. Fluorescent light photography of electroporated embryos at st. 7. C. Whole-mount ISH for TH of embryo showed in B. D. TH immunostaining after Whole-mount ISH for TH of the same embryo showed in B and C. St. 8 electroporated embryos showed approximately 2.4-fold increase in L-DOPA content measured by HPLC

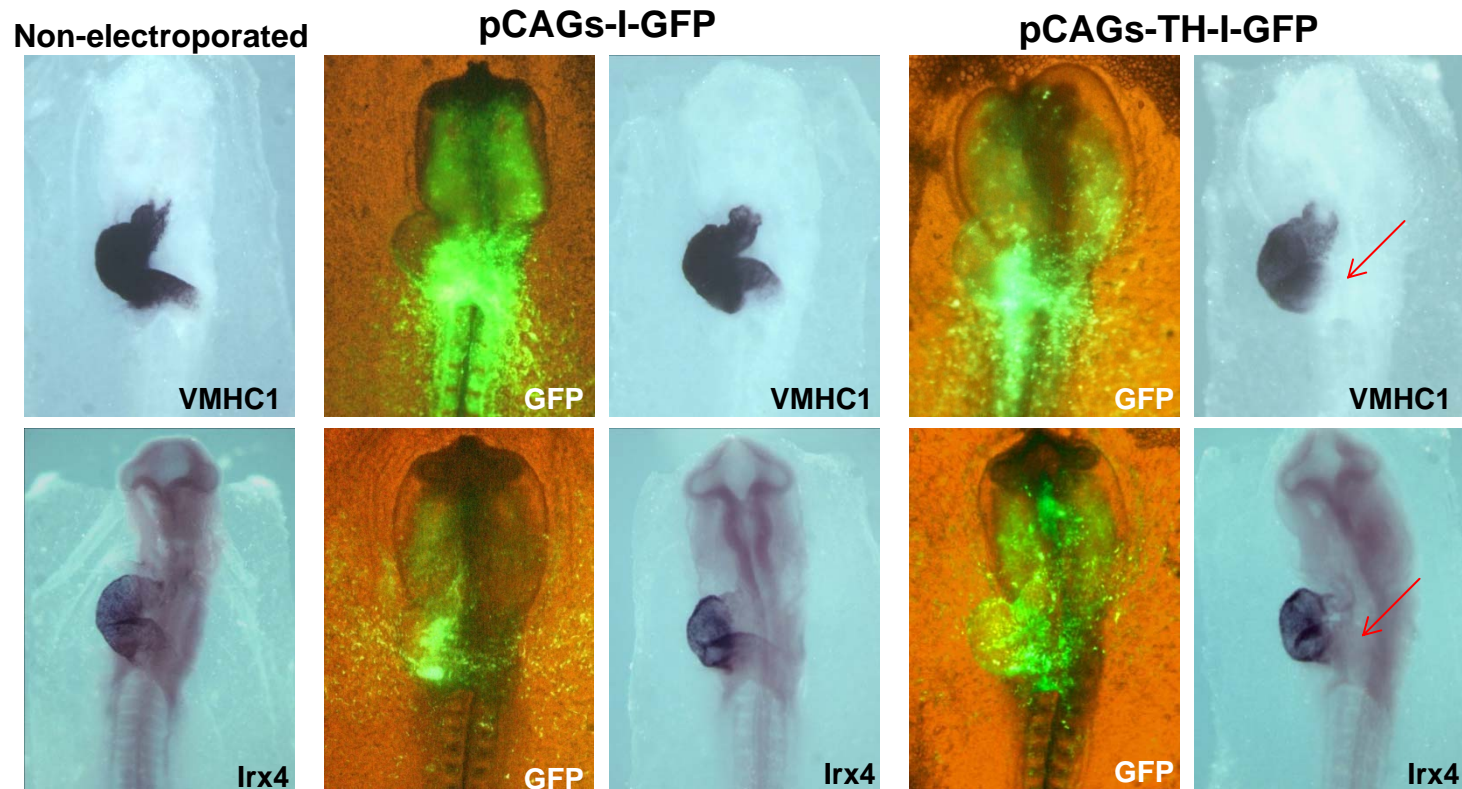


Figure S4. Effect of TH gain-of-function on ventricular gene expression. Whole-mount ISH for VMHC1 and Irx4 in non-electroporated embryos, and in embryos electroporated with either the control construct (pCAGs-I-GFP) or with the TH expressing construct (pCAGs-TH-I-GFP). Visualization of GFP expression for the embryos processed for ISH is shown in the corresponding left panels. The posterior regression of VMHC and Irx4 expression is indicated by the red arrows.

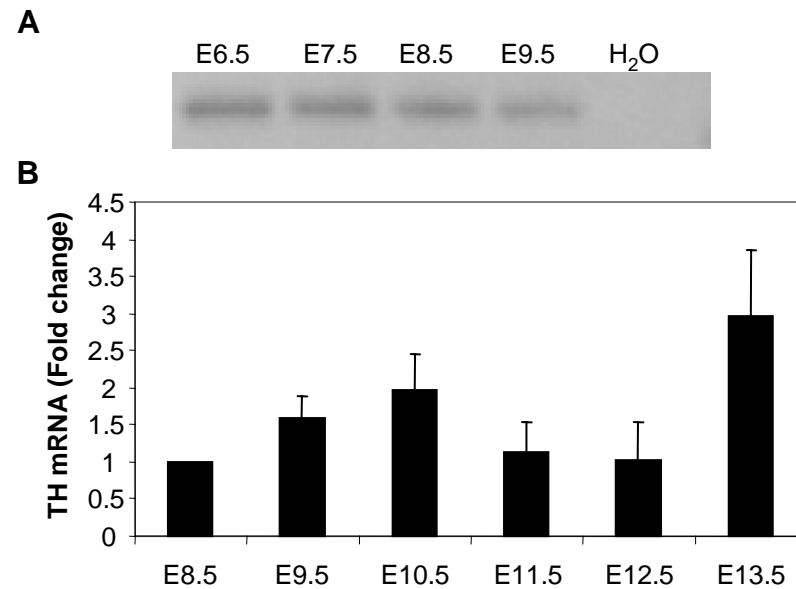


Figure S5. Expression of TH mRNA in mouse embryos. A. Standard RT-PCR of RNA from whole embryos at the indicated days of development. TH was expressed in whole mouse embryo since E6.5 (the earliest analyzed stage). B. Quantitative RT-PCR of RNA from the heart of embryos at the indicated days. The levels of TH mRNA were normalized to 18S rRNA levels. TH transcripts were detected in the mouse heart from E8.5 (the earliest analyzed stage) onwards. The results represent the mean \pm SD of two experiments.

Supplementary Material - Video, Audio

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